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The purpose and scope of ongoing studies are examination of the mechanistic involvement of c-*myb* in signaling events leading to inappropriate interpretation of growth signals in ER+/PR+ breast carcinoma. In this annual report, while early problems related to generation of high level c-*myb* expressing MCF-7 cell clones have been encountered, preliminary results indicate that transfection of c-*myb* may partly abrogate the requirement for estrogen in MCF-7 growth, a result critical to proof that c-*myb* is a critical component of estrogen signaling in estrogen responsive breast tumor cell lines. However, further work as part of the current proposal will be critical to provide definitive evidence for this. In addition, novel expression patterns of other *myb* family members along with divergent regulatory patterns indicates a unique pattern of expression for *myb* family members in breast tumor cells. These results imply a broader role than originally hypothesized for *myb* family members in breast tumor cell differentiation and proliferation and represent data critical to completion of the current project. The novel expression pattern of *myb* family members also forms an important base for a more general exploration of *myb* family members in breast development and tumorigenesis in the future.

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Eric K. Westin 9/26/97

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TABLE OF CONTENTS

FRONT COVER: page 1

STANDARD FORM (SF) 298: page 2

FOREWORD: page 3

TABLE OF CONTENTS: page 4

INTRODUCTION: page 5

BODY: page 8

CONCLUSIONS: page 10

REFERENCES: page 11

APPENDICES: page 13

INTRODUCTION:

The following represents the first annual report for IDEA grant # DAMD17-96-1-6168 entitled "Function of the myb Proto-oncogene in Breast Cancer" under contracting to Virginia Commonwealth University with Dr. Eric H. Westin as the Principal Investigator. Note that this covers the period from October 1, 1996 to July 1, 1997 at which time Dr. Westin left Virginia Commonwealth University to join the faculty at West Virginia University with current position and address as follows:

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Subject, purpose and scope of research and background of previous work:

Breast Cancer

Breast cancer represents one of the leading causes of cancer morbidity and mortality in women with an average lifetime risk of developing a primary breast tumor being one in ten. Epidemiologic studies have shown a number of factors, in addition to family history, to further increase the risk of breast tumors including early menarche, diethylstilbestrol treatment for threatened abortion and late first pregnancy. This has generally been interpreted to indicate a role for both chronic estrogen stimulation of ductal and lobular epithelial cells as well as a possible, though controversial, role of progestin in increasing an individual's lifetime risk of breast cancer(1).

Within the group of women developing breast carcinomas, considerable heterogeneity in clinical behavior exists. A number of important prognostic factors have been identified in addition to age and stage of the tumor at detection as well as histopathologic subtype and estrogen and progesterone receptor status. These include factors such as aneuploidy and high S phase fraction as determined by flow cytometric methods(2), expression of genes such as cathepsin D(3,4), as well as amplification and over expression of certain proto-oncogenes, in particular the epidermal growth factor receptor related *erbB*-2 proto-oncogene(5-7). Expression of other proto-oncogenes including the *int*-2 gene(8) and the nuclear transcription factor proto-oncogene *myc*(9) have also been associated with poor prognosis in a limited number of studies. Of particular relevance to the

present award and annual report, frequent expression of the c-*myb* nuclear transcription factor proto-oncogene has also been found in human breast tumors (10). The finding of expression of this generally hematopoietic specific proto-oncogene is unusual both in frequency of expression as well as in the correlation of this with expression of the estrogen receptor.

The c-*myb* Proto-oncogene

The c-*myb* gene was initially identified as the transforming viral oncogene in the avian myeloblastosis virus and E26 leukemia virus(11-13). The viral transforming potential has been related to the loss of N- or C- terminal sequences in the v-*myb* protein. C-*myb* is a member of the nuclear class of cellular proto-oncogenes that have been found to bind DNA (14-16) in a methylation-sensitive manner(17). Binding of the viral *myb* gene product to DNA occurs to a six base pair sequence motif(18), though additional sequences may be required to define specificity(19-21). Based on NMR analysis, the binding domain consists of a structure with a hydrophobic core of conserved tryptophan residues related to but distinct from a canonical helix-turn-helix motif(22). Multiple recent studies have shown that both the viral and cellular *myb* gene products may function as transcription activators or repressors(19,23-28). In c-*myb*, carboxyl terminal elements can play an important negative regulatory role in transcription activation(29). Possible cellular targets for positive regulation by c-*myb* include the mim-1 gene(27), the c-*myc* proto-oncogene(30-32), the *cdc2* gene(33) and the CD4 gene expressed on T lymphoid cells(34). Important to consideration of possible functions of expression of c-*myb* in breast cancers, tissue specificity of expression of transcription activation targets of *myb* may be dependent on co-expression of additional tissue specific transcription factors such as a C/EBP beta class factor involved in myeloid specific expression of the mim-1 gene(35). In addition, c-*myb* has been recently shown to repress c-*erbB*-2 transcription by direct binding to the promoter of this gene (28).

Another possible direct or indirect target of transcription activation by *myb* is the insulin like growth factor-1 (IGF-1) gene. Constitutive expression of c-*myb* in NIH3T3 mouse fibroblasts which normally express little or no c-*myb* protein, while not leading to overt transformation of these cells, does lead to loss of dependence on IGF-1 for cell proliferation. This has been shown to be due to induction of IGF-1 expression in these fibroblasts creating an autocrine response loop(36). Of interest, in breast tumors, expression of c-*myb* correlates best with expression of the estrogen receptor(10). In cell lines dependent on estrogen for growth such as MCF-7 and T47D, expression of *myb* appears to be directly regulated by exposure to estrogen based on the ability of estrogen to up regulate *c-myb* in the presence of cycloheximide (see next section, Background/Results). Exposure of these cells to estrogen has also been shown to result in induction of expression of a number of growth factors that permit continued cell proliferation even in the absence of continued estrogen exposure. Included among those critical for this proliferative response is IGF-1(37-43).

Background/Results

The concept to be tested in this "IDEA" proposal was based on the frequent observation of *myb* expression in clinical breast tumor specimens by others(10). To provide a stronger base for this proposal, three minimum conditions were felt to be necessary to justify the attempt with results provided as part of the original proposal. The first was confirmation that *myb* is expressed in a variety of breast tumor cell lines some of which are ER-/PR- and not dependent on

exogenous estrogen for growth. The second was that c-*myb* was not expressed in dividing but otherwise non-tumorigenic breast epithelium using MCF-10A as an example of a spontaneously immortalized but otherwise apparently normal growth factor responsive, non-tumorigenic, breast epithelial cell line(44). The third condition was that *myb* was expressed in an estrogen dependent manner in estrogen dependent cell lines such as MCF-7. Of interest, this regulation appeared to be in direct response to activation of the estrogen receptor since up-regulation of *myb* in response to estrogen occurred even in the presence of the protein synthesis inhibitor cycloheximide.

Hypothesis/Purpose

Analysis of expression and function of the transcription factor c-*myb* proto-oncogene has been generally limited to hematopoiesis. There it plays a major role in control of the balance of competing cell signals required for both progenitor cell expansion and programmed terminal differentiation. In surveys of proto-oncogene expression in tumors of varying histologic types, two important exceptions to the hematopoietic expression pattern have come to light: many colonic carcinomas and ER+/PR+ breast carcinomas and cell lines express *myb*. In breast carcinomas, regulation of c-*myb* expression by estradiol as part of our results and known induction of insulin like growth factor-1 (IGF-1) expression by c-*myb* suggests crossover between growth factor signaled proliferation and ER-mediated transcription events acting through the *myb* gene product. The current proposal sought to examine the mechanistic involvement of c-*myb* in signaling events which cause ER+/PR+ breast carcinoma to inappropriately interpret growth signals and the manner in which hormonal effects and growth factor signaling come together in these cells. In addition, the effect of expression of *myb* on the phenotype of breast cancer cells was also to be examined. This would provide important new insights into the mechanisms of both growth control and factors which may influence tumor behavior in breast carcinoma cells. These studies would also provide the critical information to justify more detailed studies of c-*myb* in breast cancer including examination of mechanisms regulating c-*myb* expression and elucidation of potential regulatory targets of c-*myb* that are breast tumor cell specific.

Technical Objectives of the Original Proposal

Based on the hypothesis that expression of *myb* in breast tumors and cell lines may contribute to both aberrant interpretation of estrogen induced growth signals and the phenotype of breast tumor cells in terms of tumorigenic potential, specific objectives included:

I. Determine whether c-*myb* is causally linked to the estrogen induced proliferative response observed in ER and PR expressing breast carcinoma cells. Two related experimental designs would be used to approach this central specific aim.

A. Using constitutive and regulatable expression techniques, the effect of regulation of c-*myb* on the mitogenic response to estrogen would be studied in breast carcinoma cell lines as a means of analyzing the putative control pathway.

B. Using cell lines developed in A, the effect of *myb* expression on antiestrogen responsiveness would be examined.

II. Determine whether constitutive c-*myb* expression can influence the transformed phenotype of breast tumor cell lines which do or do not normally express c-*myb*.

BODY:

Overview/Assumptions

The underlying assumption that forms the basis for the studies completed and planned for the future is that c-*myb* is causally linked to the estrogen induced proliferative response observed in ER and PR expressing breast carcinoma cells.

As part of the first 9 months of work, initial work centered on two areas of endeavor. The first was initial generation and testing on c-*myb* constitutively expressing transfectants using the MCF-7 cell line and the second was examination of expression of other c-*myb* gene family members including A-*myb* and B-*myb*(45) to determine expression patterns and whether these, as in development, were expressed in an exclusive or complementary pattern(46,47). This second avenue of work was undertaken specifically to ensure that constitutive expression experiments would be interpretable and also to determine whether some of the problems encountered in initial c-*myb* transfectants (see results below) could be logically overcome using a reverse genetic approach of dominant-negative c-*myb* variants to examine *myb* function.

Experimental Methods

i: Transfection:

Initial studies used constitutive expression vectors based on the SV40 promoter that we have utilized previously in our studies of the role of c-*myb* in FMEL cell differentiation(48). For constitutive expression of *myb*, MCF-7 cells were directly transfected and analyzed using calcium phosphate precipitation(49) and co-selection with the neomycin resistance gene and G418 (Gibco/BRL).

ii: Cell Culture/ Northern Blotting Studies:

Cells, including MCF-7, were maintained in their respective recommended medias established through the American Type Culture Collection from which all cell lines were obtained. For purposes of estrogen withdrawal and stimulation, cells were withdrawn completely from estrogen by maintenance in phenol red free media supplemented with 10% charcoal stripped fetal calf serum (Hyclone). At the time of estrogen stimulation, 1 nM beta-estradiol (Sigma) was added to this media and the time course followed as noted in the Figures and their legends.

To establish expression patterns of c-*myb* in selected transfectants as well as examine expression of A-*myb* and B-*myb*, RNA was isolated using quanidinium isothiocyanate followed by Northern blotting using procedures as described previously(50,51). Blots were probed with c-*myb*, B-*myb* and A-*myb* specific probes radiolabelled with ^{32}P by nick translation (45).

Results/Discussion

i: Transfection:

Initial transfectants of MCF-7 were established using c-*myb* under control of an SV-40 promoter with co-transfection using pSV2-neo as the selectable marker. In these transfections, selection of clones co-transfected with *myb* (as opposed to pSV2-neo alone) were problematic with expression ranging from limited to non-detectable. To overcome this problem, three

additional separate transfections were done utilizing a c-myb/DHFR (dihydrofolate reductase) construct (48) co-transfected with pSV2-neo for selection of transfectants with G418 (Gibco/BRL). This was done to permit addition selection with methotrexate of clones containing c-*myb* and to provide a potential mechanism by which c-*myb* expression could be elevated by selection of clones on increasing concentrations of methotrexate leading to gene amplification as has been done in FMEL cell hematopoietic transfectants (48). From each of these experiments, three or more initial G418 resistant transfectants containing *myb* along with controls transfected with pSV2-neo and DHFR control vector alone were analyzed. In each case, expression was again found to be limited which made functional analysis (requirement of estrogen for continued growth of clones, see Figure 1 in the Appendix) difficult to interpret. In addition, co-selection on increasing concentrations of methotrexate (either using individual cell clones or pools from two additional transfections) did not lead to outgrowth of selectable clones expressing significant levels of *myb*.

Since development of clones expressing exogenous *myb* is critical for testing of the central hypothesis, one question raised by results to date was whether exogenous expression of c-*myb* using and SV-40 promoter system was in some way toxic to MCF-7 cells indicating the need to move directly to a strictly regulated system proposed as a subsequent part of the initial proposal. To partially address this, two quantitative transfections were done with MCF-7 and colonies quantitated after 3 weeks of growth on selective (G418 containing) media. In these experiments, the ratio of c-*myb* vector to selectable marker was kept at 10:1 in the calcium phosphate precipitation, in which case over 70% of clones would be likely to contain the c-*myb* vector. In these transfections, compared with controls of selectable marker alone, no difference was seen in colony number indicating that the problem was not strictly one of an unknown "toxic" effect of *myb* expression on these MCF-7 cells but rather an unexpected technical problem related to the constructs chosen for transfection and analysis.

Given this data to date, it is clear that alternative transfection methods will be required to complete this portion of the proposal. As proposed in the original proposal, at minimum two alternative approaches will be utilized if subcontracting is arranged from Virginia Commonwealth University to West Virginia University. As originally proposed, should problems be encountered in obtaining adequate levels of constitutive c-*myb* expression using SV40 promoter/enhancer based constructs, the human elongation factor-1 promoter (pEF-BOS) would be tested(52) as an alternative promoter reported to permit high constitutive expression in transfected cells. Since the problems to date do not appear to represent a direct toxic effect of *myb* on MCF-7 cells, this remains an attractive approach. In addition, the tetracycline repressible promoter expression constructs available in the laboratory would also attempted(53,54). Finally, with the move to West Virginia University, another attractive approach now available given availability of appropriate interacting investigators from which vectors and techniques can be obtained is the use of an amphotrophic retrovirus to infect rather than transfect MCF-7 cells. Use of this approach would provide and additional alternative for development of either larger numbers of higher level exogenous c-*myb* expressing clones or permit an alternative approach to use of pools of clones eliminating the need to analyze 5-10 individual independent clones to overcome possible artifacts related to integration of DNA in either transfection or retroviral mediated gene transfer experiments. Because of findings with expression of both A-*myb* and B-*myb* (see next section), while an additional alternative of using dominant-negative forms of c-*myb* might have represented a logical alternative given initial preliminary results from the original proposal, this cannot be

considered useful at present since multiple family members of *myb* are co-expressed in breast tumor cell lines. This makes interpretation of effects of a dominant-negative c-*myb*, with its potential ability to antagonize effects of all *myb* family members due to similar DNA binding properties, impossible.

ii: A-*myb* and B-*myb* expression

As discussed in the introduction, examination of expression of other c-*myb* gene family members including A-*myb* and B-*myb*(45) was undertaken to determine expression patterns and whether these, as in development, were expressed in an exclusive or complementary pattern(46,47). This second avenue of work was undertaken specifically to ensure that constitutive expression experiments would be interpretable and also to determine whether some of the problems encountered in initial c-*myb* transfectants (see above) could be logically overcome using a reverse genetic approach of dominant-negatives to examine c-*myb* function. From this work, shown in the Figures 2-4 in the Appendiz, B-*myb* is expressed in all breast tumor cell lines examined and is not regulated in response to estrogen in MCF-7. In contrast, A-*myb* is regulated in parallel with c-*myb* including its dependence on estrogen receptor stimulation in MCF-7. This represents a unique pattern of expression for *myb* family members, with a cell line such as MCF-7 expressing all three family members simultaneously and implies a much broader role for *myb* family members than originally hypothesized in breast tumor cell differentiation and proliferation. It also indicates that the function of each family member (c-*myb* in the case of the current funded project, A-*myb* and B-*myb* in potential future work) will have to be dissected through use of constitutive expression and/or specific anti-sense techniques, avoiding the use of an otherwise attractive dominant-negative approach.

Recommendations

Based on the results and discussion above as well as data provided in the Appendix, while initial problems were encountered in generating the transfectants needed to provide proper testing of the hypothesis that c-*myb* is integral to estrogen stimulated breast tumor cell proliferation, significant progress has been made. This includes both early results that indicate that the hypothesis may be correct (and therefore critical in our understanding of breast tumor cell proliferation) and provision of the data needed for design of a subsequent series of vectors as discussed to achieve the original statement of work. In addition, novel expression patterns of other *myb* family members along with divergent regulatory patterns indicates a unique pattern of expression for *myb* family members, with a cell line such as MCF-7 expressing all three family members simultaneously. This implies a much broader role for *myb* family members in general than originally hypothesized in breast tumor cell differentiation and proliferation. As discussed, this work also indicates that the function of each family member (c-*myb* in the case of the current funded project, A-*myb* and B-*myb* in potential future work) will have to be dissected through use of constitutive expression and/or specific anti-sense techniques, avoiding the use of the otherwise attractive dominant-negative approach.

CONCLUSIONS:

While early problems related to generation of high level c-*myb* expressing MCF-7 cell clones has been encountered, very preliminary results indicate that transfection of c-*myb* may in

part abrogate the requirement for estrogen in MCF-7 growth, a result critical to proof that c-*myb* is indeed an important component of estrogen signaling in estrogen responsive breast tumor cell lines. However, further work as part of the current proposal will be critical to provide definitive evidence for this. In addition, novel expression patterns of other *myb* family members along with divergent regulatory patterns indicates a unique pattern of expression for *myb* family members in breast tumor cells, with a cell line such as MCF-7 expressing all three family members simultaneously. These results imply a much broader role than originally hypothesized for *myb* family members in breast tumor cell differentiation and proliferation and represent data critical to completion of the current project. The novel expression pattern of *myb* family members also forms an important base for a more general exploration of *myb* family members in breast development and tumorigenesis in the future.

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APPENDICES:

Figure Legends:

Figure 1: MTT assay of transfected MCF-7 cell clones in the presence and absence of estrogen. 1.0: MCF-7 parent cell line; 2.0-4.0: pSV2-neo alone control transfectants of MCF-7; 5.0-7.0: c-*myb* transfectants of MCF-7. For this experiment, individual cell clones were withdrawn from estrogen and then stimulated for the indicated time periods with 1 nM beta-estradiol (Sigma). Note that though there is a tendency for estrogen independent growth in c-*myb* transfectants compared with MCF-7 parental or pSV2-neo controls, this not entirely consistent (see clone 3.0) and therefore, while "suggesting" that the initial hypothesis that c-*myb* is linked to the estrogen induced proliferative response my be correct, is by no means sufficient at this point to provide full proof for this hypothesis.

Figure 2: Northern blot of c-*myb* expression in the breast tumor cell lines as indicated in the figure.

Figure 3: Northern blot of B-*myb* expression in the breast tumor cell lines as indicated in the figure.

Figure 4: *Myb* family member expression in response to withdrawal of estrogen and restimulation in MCF-7. MDA-231: c-*myb* non-expressing control breast tumor cell line (note expression of both A-*myb* and B-*myb* however); MCF-7: MCF-7 under normal estrogen dependent growth conditions; -E: MCF-7 after 7 days of estrogen withdrawal; +E (estrogen, 1nM beta-estradiol), +C (cyclohexamide), +E,+C (estrogen and cyclohexamide treated): MCF-7 exposed as indicated for 18 hours. Probes used were as indicated. GAPDH (Glyceraldehyde Phosphate Dehydrogenase) is included as a control of RNA loading.

Figure 1:

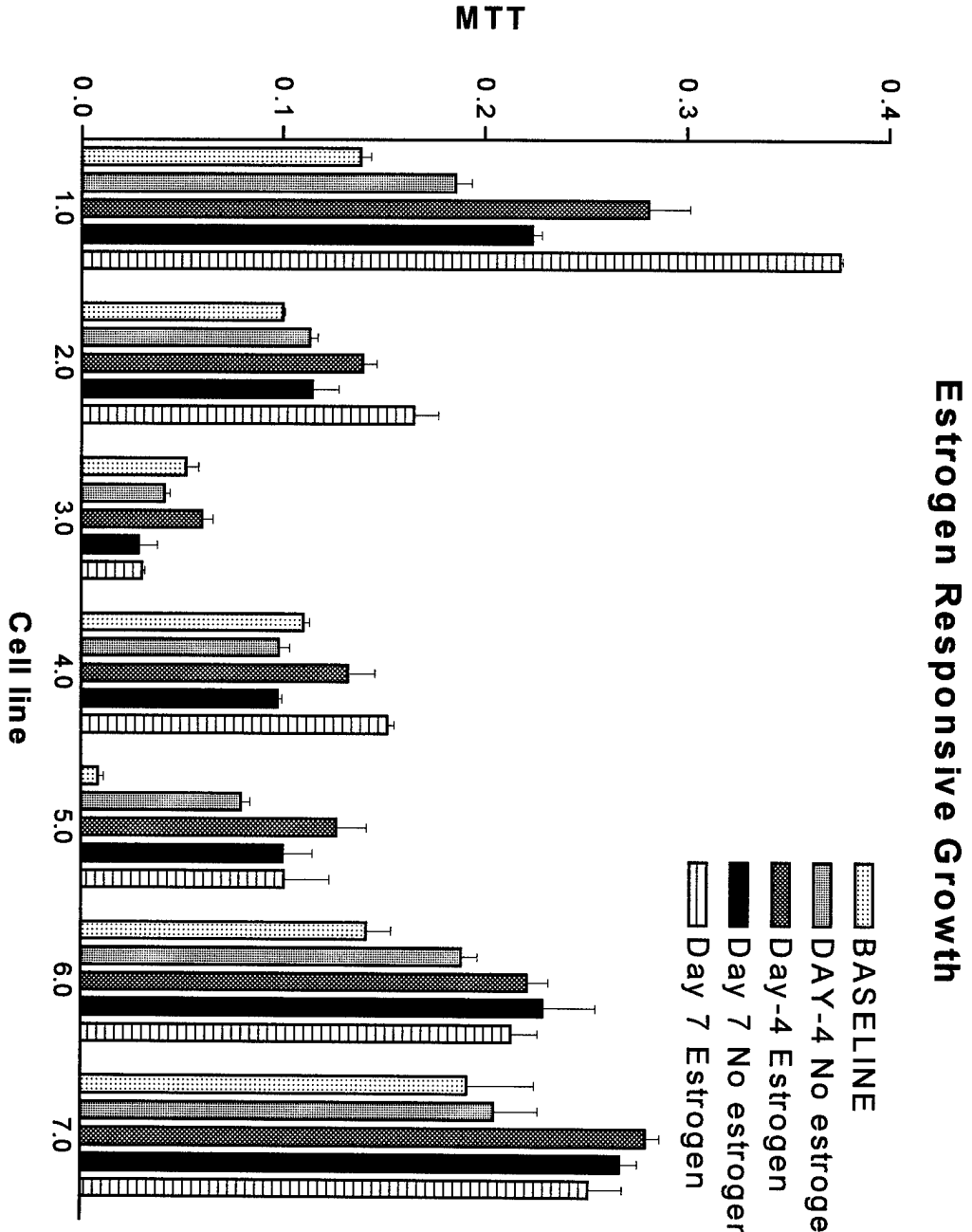
MTT
0.0
0.1
0.2
0.3
0.4
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Cell line
Estrogen Responsive Growth
BASELINE
DAY-4 No estrogen
Day-4 Estrogen
Day 7 No estrogen
Day 7 Estrogen

Figure 2:

c-myb

BT-20

BT-594

BT-774

DU-4475

MDA-361

MDA-453

MDA-468

SKBR3

T47D

ZR75-1

MCF-7

Figure 3:

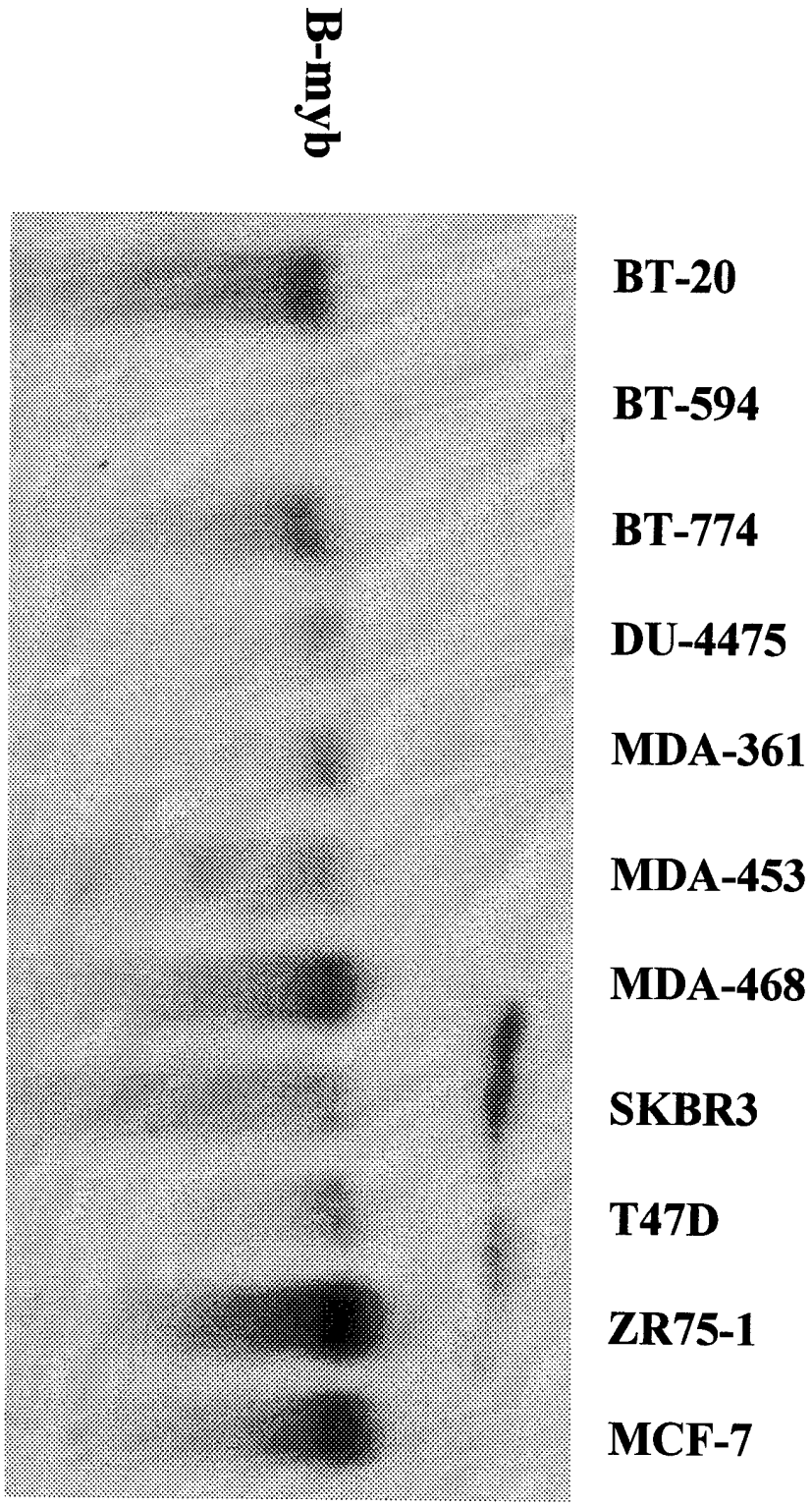
B-myb
BT-20
BT-594
BT-774
DU-4475
MDA-361
MDA-453
MDA-468
SKBR3
T47D
ZR75-1
MCF-7

Figure 4: